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Effect of butyrate on the expression of the human preprourokinase gene introduced into Chinese hamster ovary cells.
Okabayashi K, Kaneda T, Arimura H.

2) J Biotechnol 1991 Jun;19(1):35-47
Production of analytical quantities of recombinant proteins in Chinese hamster ovary cells using sodium butyrate to elevate gene expression.
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Thank you,
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Production of analytical quantities of recombinant proteins in Chinese hamster ovary cells using sodium butyrate to elevate gene expression

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Summary

Sodium butyrate was used to enhance expression levels and thereby facilitate the generation of analytical quantities of nine different tissue plasminogen activator (tPA) analogues expressed under the control of the cytomegalovirus immediate early (CMV IE) promoter by the Chinese hamster ovary (CHO) mammalian expression system. Production involved growth in roller bottles, using serum free or low serum media formulations, together with repetitive, sodium butyrate inductions. Average inductions in the presence of sodium butyrate ranged from 2 to 9-fold relative to uninduced controls, using cell lines with no previous butyrate exposure. Retardation of growth rate by butyrate minimized the need to split cells during the production runs, extending longevity of roller bottles containing cells secreting at induced levels. SDS-PAGE analyses indicate a consistently high percentage of single-chain material. Measurements of specific activity and fibrinogen fragment enhancement for one of the analogues demonstrate that neither of these two critical parameters are affected by production in the presence of butyrate. Induction kinetic data and growth curves for the expression of sCD4 under control of the SV40 early promoter demonstrate that the benefits of butyrate can be realized with different promoters and heterologous genes, and are additive when used in conjunction with an amplified cell line constitutively expressing at elevated levels. This work demon-

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strates the practical application of sodium butyrate in the production of analytical quantities of protein from the CHO expression system, and suggests a role for sodium butyrate in commercial scale processes as well.

Mammalian expression; CHO; Sodium butyrate; Gene induction

Introduction

Preliminary analyses of heterologous proteins synthesized by an expression host typically don't require large amounts of protein. Rather, rapid access to moderate amounts, (several hundred micrograms to several milligrams) of high quality material is of primary importance.

Oftentimes, however, expression levels are initially low, and methotrexate-mediated gene amplification can be slow. These factors impede the use of mammalian expression systems in situations where the primary focus is on rapid analysis of the recombinant protein and related analogues.

In 1983, Gorman et al. (1983) showed that HeLa or monkey kidney CV-1 cells that are stably transfected in the presence of sodium butyrate with a recombinant chloramphenicol acetyltransferase (CAT) gene under the control of the SV40 early promoter respond to subsequent butyrate exposure with up to a 7.5-fold induction of CAT expression. Puddington et al. (1987) used sodium butyrate to induce detectable quantities of VSV G protein under control of the RSV promoter. Kooistra et al. (1987) demonstrated that butyrate induces expression of endogenous tissue plasminogen activator from cultured human endothelial cells. Dorner et al. (1989) demonstrate an increase in secretion of Factor VIII, Von Willebrand factor and erythropoietin in response to exposure of stable transfectants to sodium butyrate. Thus it seemed that sodium butyrate might be used to rapidly increase basal expression rates of stably transformed cell lines to levels which would simplify the generation of sufficient protein for analytical studies, without necessarily having to resort to time-consuming gene amplification and cell line development strategies.

This report describes the effects of sodium butyrate on cellular growth rate, as well as on the expression rate and fidelity of the recombinant protein produced under the influence of sodium butyrate. We demonstrate that the expression enhancement effect is a general one, working for sCD4 under control of the SV40 immediate early promoter/enhancer, as well as tPA analogues under control of the CMV IE promoter. Additionally, we show that an enhancement can be obtained using amplified cells, and that no previous exposure to sodium butyrate (e.g. during transfection) is necessary. Lastly, we describe a repetitive sodium butyrate induction regimen which allowed for the production of analytical quantities of multiple tPA analogues using low or serum-free media formulations.

Materials and Methods

Cells and expression constructs

tPA analogues and the sCD4 gene were incorporated into a pSV2dhfr-based expression vector (Subramani et al., 1981) using the CMV immediate early promoter and the SV40 early promoter respectively, and the bGH polyadenylation signal sequence as described (Poorman et al., 1986; Rehberg et al., 1989). CHO dhfr$^-$ cells served as expression host (Urlaub and Chasin, 1980). All cells were maintained in a 37°C incubator with a 5% CO_2 and 95% relative humidity atmosphere.

Various tPA gene constructs were used. Five of the cell lines had undergone methotrexate-mediated gene amplification (see below).

CHO cell transfections

Transfections in the absence of sodium butyrate were based on the protocol of Graham and van der Eb (1973). Exponentially growing cells were trypsinized using a solution of 0.25% trypsin (no EDTA) in 1 × PBS (Gibco) 24 h prior to transfection. Approximately 5×10^6 cells were seeded into each T25 flask (all plasticware from Corning) to be transfected. Three h prior to addition of the DNA precipitate, spent media was replaced with fresh media. Media for non-transfected CHO cells consisted of Ham's F12 nutrient mixture supplemented with 10 mM Hepes, 100 units ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and 10% heat inactivated fetal bovine serum (all from Gibco). Host cells for transfection were carried for no more than 10–15 passages, after which a new vial of cells was thawed.

Precipitates were formed as follows. One μg of plasmid DNA, typically in from 1–10 μl sterile TE (TE is 10 mM Tris pH 8.0, 1 mM EDTA) and 31 μl of 2 M CaCl were added to 250 μl TE plus carrier DNA (10 mM Tris pH 8.0, 1 mM EDTA, carrier DNA at 5 mg ml^{-1}) in a sterile plastic tube. This mixture was removed to a second tube containing 250 μl 2 × HBS (2 × HBS is 0.28 M NaCl, 0.05 M Hepes, 3 mM Na_2HPO_4; pH 7.1, filter sterilized; stored at 4° C). Further mixing was by gentle aeration. The precipitate was allowed to form for 30 min at room temperature before it was added to the cells. The cells were incubated with the precipitate under a 5% CO_2, humidified atmosphere at 37°C for between 4–8 h.

After the incubation period, the medium containing the DNA precipitate was removed and 1.5 ml of a 15% glycerol/HBS solution (room temperature) was added to each flask (Lopata et al., 1984). Cells were immediately placed at 37°C for 1 min after which the glycerol was diluted with 5 ml Ham's medium (as described above) and quickly removed. Cells were washed 3 times with 5 ml Ham's medium, and finally fed with 5 ml Ham's per T25 flask. The transfected cells were incubated at 37°C for 48 h after which they were trypsinized and passed to a T150 flask containing selective medium (high glucose (4500 mg l^{-1}) DMEM plus glutamine supplemented with 10% heat inactivated fetal bovine serum, 0.1 mM MEM nonessential amino acids, 10 mM Hepes, pH 7.3, 100 units penicillin per ml and 100 mg

ml^{-1} streptomycin: all media and supplements supplied by Gibco). $dhfr^+$ cells appeared as foci within 7–10 d after seeding into selective medium.

Transfections which included sodium butyrate (Sigma Cat. No. B-5887) were identical except that butyrate was added to 5 mM during the adsorption of the $CaPO_4$-DNA precipitate. All subsequent steps were as described above.

Amplification

Cells were adapted to growth in 100 nM MTX (Lederle) essentially as described by Kaufman et al. (1985). Pools of transfectants were seeded at about 80% confluency into T150 flasks in selective medium (see above) plus 100 nM MTX. The medium was changed every 3 to 4 days. At confluency, cells were split 1 : 10. After three successive passages, amplification was evaluated by quantitation of product present in culture supernatants. Cells which had successfully undergone amplification were subjected to adaptation at higher MTX concentrations.

Butyrate induction

All butyrate induction experiments were conducted on cells which had survived the selection procedure after transfection (stable transfectants). Cells transfected with various tPA analogues were exposed to butyrate after growing to approximately 90% confluency in T75 flasks. Spent medium was removed and fresh medium minus butyrate was added. Uninduced (control) conditioned culture medium was harvested for assay 24 h later. The same monolayers were then refed with fresh medium containing 5 mM butyrate (Sigma No. B-5887). Butyrate-containing, conditioned culture medium was harvested 24 h later and assayed for plasminogen activator as described below.

Long term butyrate inductions

Long term butyrate inductions were performed as follows. Cells secreting each of nine analogues were seeded into duplicate roller bottles (850 cm^2, Corning) and grown to 90% confluency. Monolayers were washed with 1 × PBS to remove residual serum, and fed (75 ml per bottle) with serum free media formulated as follows; high glucose DMEM mixed 1 : 1 with MCDB 301 (Gibco; MCDB formula No. 78-0037A5) supplemented with 5 $\mu g\ ml^{-1}$ each of insulin, transferrin, and selenium ("ITS"; Collaborative Research No. 40351), 0.1 mM MEM non-essential amino acids, 10 mM Hepes, pH 7.3, 100 units penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 2.0 mM glutamine (all from Gibco), 1 $\mu g\ ml^{-1}$ aprotinin (Boehringer Mannheim No. 981-532), 10 mM epsilon amino caproic acid (EACA; Sigma No. A-2504) and 5 mM sodium butyrate. Amplified lines usually required 3% FBS for best growth and production, and were supplemented with a final concentration of methotrexate (MTX: Lederle No. NDC 0205-5325-26) appropriate for the individual lines.

Cells were incubated in the presence of 5 mM sodium butyrate for 48 h, at which time conditioned culture medium was harvested, cleared by centrifugation at $7000 \times g$ for 10 min, assayed and stored frozen. The cells were refed with butyrate-containing medium, and incubated for an additional 48 h. Conditioned supernatants were again harvested, cleared, assayed and pooled with previous harvests. Cells were then fed with standard selective medium plus 10% FBS for 72 h. The induction-harvest cycle was repeated, typically for a period of 3–4 weeks, or until product secretion tailed off significantly.

Growth curves and kinetics of butyrate-mediated induction

T25 flasks were seeded at a cell density of about 4×10^5 cells per flask from a common suspension consisting of cells transfected with the sCD4 expression construct. This clonally derived line had been adapted to growth in 500 nM MTX, and had demonstrated about a 10-fold increase in secretion of sCD4 relative to its unamplified parental clone. Eighteen h after seeding, fresh selective medium (as described above) ± 5 mM butyrate was added to the flasks, 0 h supernatants were harvested, and cell counts determined by hemocytometer. At various intervals over the next 72 h, supernatants were harvested and cell counts determined. Supernatants were assayed for sCD4 as described below.

Assays

tPA activity (measured in International Units) was assayed by colorimetric activity assay as described by Verheijen et al. (1982).

Active site titrations were performed for tPA using ^{3}H-DFP according to the method of Rehberg et al. (1989).

Fibrin activation studies were performed both before and after plasmin cleavage of single chain tPA (Rehberg et al., 1989) using fibrinogen fragments prepared according to the method of Verheijen et al. (1982).

Levels of sCD4 were determined by Particle Concentration Fluorescence Immunoassay (PCFIA). Each assay utilized two monoclonal antibodies (mAbs) which recognize distinct epitopes of sCD4. A solid phase (polystyrene particle attached) mAb captured sCD4 and another mAb (fluorescein tagged) marked the molecule. Reactions were carried out in special 96-well plates equipped with conical membrane-bottomed wells and a vacuum chamber underneath. Once sCD4 was sandwiched between capture mAb and marker mAb vacuum was applied and unbound fluorescein-tagged mAb was filtered away from the solid phase leaving only marker antibody that was attached to sCD4. The signal from this complex was directly proportional to the concentration of sCD4 present which could be interpolated from a calibration curve of standard sCD4. Full length extracellular sCD4 (CD4ex1 from E. Rienherz, Harvard Univ.) was the standard for all assays.

Phase separation and fluorescence measurements were done with a FCA (Fluorescence Concentration Analyzer). The analyzer, assay particles, and assay plates were purchased from Pandex/Baxter Scientific.

The assay utilized capture antibody UCCD4b (Upjohn) bound to polystyrene particles. The marker antibody (OKT4-FITC) was purchased from Ortho Diagnostics. UCCD4b has specificity for domain I of the sCD4 molecule and OKT4 is specific for a region between domains III and IV.

Capture antibody and sample (50 μl) were incubated at room temperature for 20 min. Marker antibody was then added and the incubation continued for 15 min. The unbound OKT4-FITC was then aspirated away and the signals from the bound conjugates were compared to a calibration curve which extended from 0.8–200 ng ml^{-1}. Cell supernates were assayed at dilutions ranging from 1 : 5 through 1 : 500 without non-specific interference.

Analogue purification and SDS-PAGE

Analogues were purified from conditioned culture supernatants as described (Rehberg et al., 1989). SDS-PAGE was performed as described by Maniatis et al. (1982).

Results

tPA analogues: characterization of butyrate inductions

Gorman et al. (1983) suggested that butyrate had to be present during the transfection in order to obtain an induction of heterologous gene expression in response to subsequent butyrate exposure. Initially, we wanted to determine whether a response to sodium butyrate could be obtained with cells transfected in the absence of sodium butyrate.

Expression vectors representing 6 different tPA analogue constructs were used to transfect CHO cells both in the presence and absence of 5 mM sodium butyrate, for a total of 12 individual transfections. Stable transfectants were selected and the twelve cultures were re-exposed to 5 mM butyrate. The averages of duplicate inductions are shown in Table 1. With inductions ranging from 2 to 9-fold, butyrate responsiveness was readily observable regardless of whether the cells had seen butyrate during the transfection.

Biochemistry of butyrate-induced tPA

Native tPA and the analogue FGK_1K_2P were synthesized $\pm$ butyrate to determine whether synthesis in the presence of butyrate changed two significant biochemical properties of the molecules. Specific activity and fibrinogen fragment enhancement factors were compared, before and after plasmin-cleavage to the two chain form. Data appear in Table 2. With regard to these parameters, tPA produced in the presence of butyrate is not significantly different from its uninduced counterpart.

TABLE 1

Analogue	tPA expression		Fold induction
	Without IU per 10^6 cells per day	With IU per 10^6 cells per day	
FK_2P	52	227	4.4
FK_2P *	43	132	3.1
FK_1K_2P	36	116	3.2
FK_1K_2P *	97	235	2.4
FGK_1P	4	8	2.0
FGK_1P *	2	7	3.5
FGK_1K_2P	56	159	2.8
FGK_1K_2P *	33	304	9.2
FGK_2K_1P	16	71	4.4
FGK_2K_1P *	13	81	6.1
GK_1K_2P	2	7	3.5
GK_1K_2P *	3	7	2.3

* Cells transfected with 5 mM sodium butyrate present during DNA uptake. Butyrate-responsiveness of stable cell lines transfected either in the presence or absence of sodium butyrate. Each of six different tPA analogues were cloned into expression constructs utilizing the CMV immediate early promoter (Materials and Methods). Each construct was then transfected into CHO cells either with (*) or without butyrate exposure during the transfection protocol (Materials and Methods). Twelve stable cell lines representing each of the 6 constructs transfected under the 2 conditions were clonally derived. To compare the response of each to subsequent butyrate exposure, each of the lines was subsequently exposed to 5 mM butyrate. Cells were grown in duplicate flasks to 90% confluency, washed, refed and conditioned culture medium was harvested 24 h later (representing expression observed minus butyrate). Cells were again washed and refed, this time with medium supplemented with 5 mM butyrate. Induced conditioned culture medium (representing expression plus butyrate) was harvested 24 h later, cell numbers determined by hemocytometer. Control flasks receiving fresh medium without butyrate after 24 h showed no measurable difference in rate of tPA secretion between the first 24 h harvest and the second (data not shown).

TABLE 2

Plasminogen	Specific activity IU mg^{-1}	
	+ Butyrate	− Butyrate
FGK_1K_2P	461 225 ± 3 300	479 472 ± 3 400
Native tPA	591 720 ± 2 400	539 533 ± 9 430
Enhancement factor		
FGK_1K_2P	48 ± 11	37 ± 2
Native tPA	108 ± 25	126 ± 10

Relative specific activity (IU per mg protein) and fibrin enhancement of tPA analogues made in the presence and absence of sodium butyrate. The specific activity of each tPA sample was calculated as the ratio of activity to antigen as determined by DFP-titration. The ability of the analogues to demonstrate an enhancement of activity (enhancement factor) in the presence of fibrinogen fragments was determined as follows. Activity of tPA at three concentrations (10, 20 and 30 pM) was measured spectrophotometrically over time (assay utilized substrate p2251 from Helena Labs No. 5277). A slope representing the absorbance change over $time^2$ was calculated. These slopes were plotted against concentration, representing the absorbance change per $time^2$ per molar concentration of enzyme. The ratio of the slope in the presence of fragments to the slope in the absence of fragments is the enhancement factor.

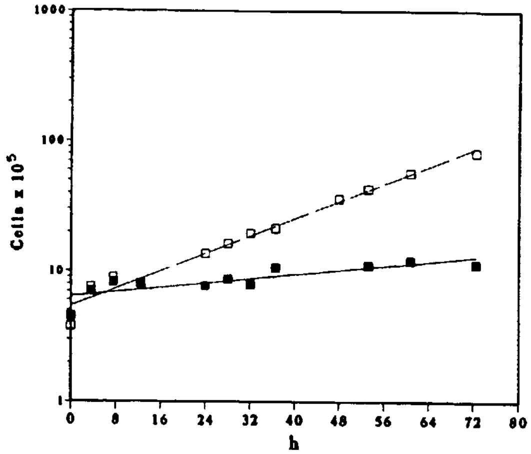

Fig. 1. Effects of sodium butyrate on growth rate. A clonally derived, highly amplified cell line secreting high levels of the T-cell receptor sCD4-369 expressed from the SV40 early promoter was used to determine the effects of butyrate on growth rate. Cells were grown in selective media containing 10% fetal bovine serum and 500 nM MTX (Materials and Methods). ■ + butyrate; □ − butyrate.

Effects of sodium butyrate on growth and secretion rates

It is well documented that sodium butyrate reversibly arrests cells at the G1 to S phase transition point of the cell cycle (Kruh, 1982; Prasad and Sinha, 1976; Chabanas et al., 1985; Parker et al., 1986). The cells stop dividing and uptake of

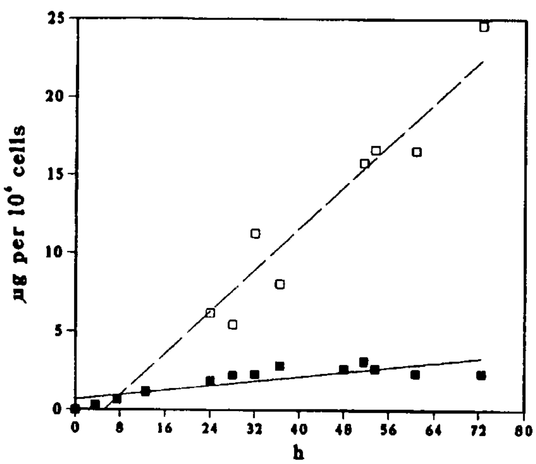

Fig. 2. Effects of sodium butyrate on the expression of sCD4-369. Supernatants were harvested during the growth curve experiment described in Fig. 1 and assayed for sCD4-369 (Materials and Methods). ■ − butyrate; □ + butyrate.

TABLE 3

Construct	Line	Amplification	Total yield (μg)
FGK_1K_2P	12B/100–13	100 nM MTX	9600
FK_2P	BF500–11	500 nM MTX	4700
FGK_1P	11R–13/100–13	100 nM MTX	10620
FGK_2K_1P	15–2/100–12/500–3	500 nM MTX	511
FK_2K_2P	14–2	–	210
$FFGK_1K_2P$	27–2	–	501
FGk_1K_2P	26–1	–	585
FGk_1P	19–8	–	311
cDNA	12/500–2	500 nM MTX	27000

Application of repetitive butyrate induction in the production of analytical quantities of various tPA analogues. Analogue constructs listed above (Rehberg et al., 1989) were cloned into the expression construct described in Materials and Methods, and transfected into CHO cells. The lines were then subjected to the repetitive butyrate induction regimen described in Materials and Methods. Each line is clonally derived. Those adapted to growth in the indicated concentrations of MTX are presumed to have undergone gene amplification, based on elevated expression levels (on a per cell per day basis) relative to parental lines. Yields presented are total yields for the experiment. Expression on a per cell per day basis in the roller bottles was not determined.

[^{3}H]thymidine into DNA ceases, yet protein synthesis continues, in some cases at an enhanced level (Boffa et al., 1981; Hagopian et al., 1977; Daniell, 1980). Wright (1973) demonstrates a reversible increase in doubling time for CHO from 14 to 33 h. Our data confirms this inhibitory effect for sCD4-secreting cells (Fig. 1). Fig. 2 demonstrates that despite this growth arrest, rate of secretion far outpaces that from cells not exposed to butyrate.

Practical application of butyrate induction

Butyrate's ability to both arrest cell division and increase gene expression presents obvious advantages for production. In the following experiment, butyrate's ability to reduce growth rate allowed for minimal handling of cells during the production runs while increasing the productive life of the high density cultures. The ability of butyrate to enhance expression levels reduced the quantity of cells needed to generate the required amount of protein.

Inocula of cells to be used for production were seeded into duplicate roller bottles and cycled through a repetitive regimen of butyrate induction followed by a period of recovery as described in Materials and Methods. Table 3 demonstrates the yields achieved from two roller bottles per analogue over a one month production run. As can be seen, total yields of various analogues ranged from about 200 μg to over 27 mg.

A preliminary evaluation of product quality produced under these conditions was determined by observing the degree of degradation to two-chain material via SDS-PAGE analysis. Analogue was purified from conditioned culture supernatant and electrophoresed on 12% Laemmli gels as described in Materials and Methods.

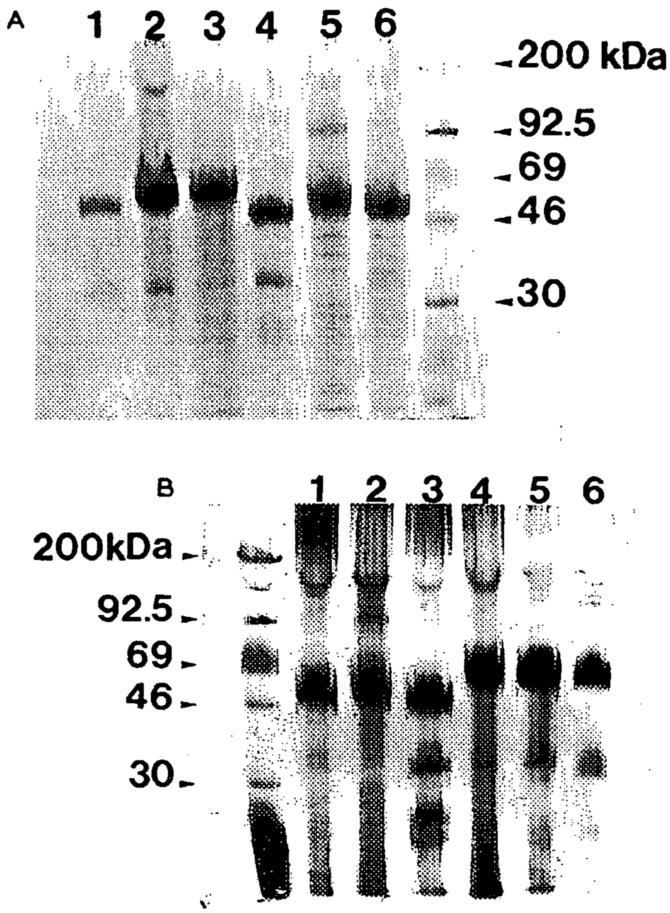

Fig. 3. Gel analyses of purified analogues. Panel A; lane 1; cDNA: lane 2; FGK_1K_2P: lane 3; FGk_1K_2P: lane 4; FGK_1P: lane 5; FK_2K_2P: lane 6; FGk_1P. Panel B; lane 1; FGk_1P: lane 2; FK_2K_2P: lane 3; FGK_1P: lane 4; FGk_1k_2P: lane 5; FGK_1K_2P: lane 6; cDNA. A = Coomassie; B = silver.

Gels were either Coomassie or silver stained, and photographed. Data are presented in Fig. 3.

The Coomassie-stained gel (Panel A) shows predominantly single-chain material for the six analogues analyzed. The more sensitive silver-stained gel (Panel B) reveals the presence of a small percentage of two-chain material amongst the analogues produced in the presence of serum (UG-3, UG-4, UG-6, UG-9). Those analogues generated in the absence of serum (UG-29 and UG-24) possessed no detectable two-chain material at all.

Discussion

Although butyrate's mechanism of action is unknown, it is clear that butyrate is capable of causing changes in chromatin structure as a result of hyperacetylation of histones, and that these changes can be correlated with a modulation of gene expression (Kruh, 1982). Our data (Table 1) demonstrate that inductions as high as 9-fold can be achieved in response to growth in butyrate, and that, on average, fold induction is not influenced by presence or absence of butyrate during the transfection when gene expression is under control of the CMV IE promoter. Thus, we were able to exploit the benefits of butyrate-enhanced expression levels without having to re-transfect the large number of analogue expression vectors with which we were working.

Additionally, cells stably transfected in the absence of butyrate, and expressing the T-cell receptor CD4 under control of the SV40 early promoter responded to butyrate with a cessation of growth (Fig. 1) while increasing secretion 10-fold relative to uninduced controls (Fig. 2). This very reproducible result is in apparent contrast to the results of Gorman and Dorner (Gorman et al., 1983; Dorner et al., 1989). While we have observed clonal variations in fold induction in response to butyrate exposure, we have repeatedly been able to achieve an induction of protein secretion for genes under control of the SV40 promoter, regardless of the cells' previous butyrate exposure history (data not shown).

We conclude, therefore, that changes in chromatin structure that may occur in response to exposure to butyrate need not necessarily occur initially during the integrative process at transfection in order for stable transfectants to respond to subsequent butyrate exposure with an induction of expression of these transfected genes. Additionally, these data suggest that butyrate-mediated induction of gene expression is likely to be a general phenomenon, showing little preference for promoter or gene.

Data in Fig. 2 demonstrates a 10-fold induction of sCD4-369 in response to butyrate exposure. Being an amplified line (secreting 10-fold more sCD4-369 on a per 10^6 cells per day basis relative to its unamplified parent; data not shown), this result suggests that induction and gene amplification can be additive, a conclusion also supported by data appearing in Table 3.

Data in Table 2 address whether protein synthesis in the presence of butyrate induces any measurable changes in the molecules' basic biochemistry. The data show that neither specific activity nor fibrinogen fragment enhancement, two parameters which would be expected to reflect changes in protein conformation, change significantly for molecules produced in the presence or absence of butyrate.

These results suggested the use of sodium butyrate in small scale production of tPA analogues for analytical use. The ability of butyrate to arrest cell growth was exploited to extend the productive life of high density cultures expressing at induced levels. Table 3 demonstrates the yields achieved from two roller bottles per analogue over a one month production run. The yield advantages afforded by MTX-mediated amplification in conjunction with butyrate are apparent from this table.

The quality of the protein produced in this fashion was evaluated via SDS-PAGE

(Fig. 3). The material generated by this technique is predominantly single-chain. While this is a very preliminary evaluation of the quality of the product, it does provide some insight into the degree of degradation ongoing in the production/ purification process. While N-terminal analyses would provide definitive data concerning the proteins' integrity, material that is single chain is considerably less likely to be clipped at other locations (data not shown).

That the small degree of degradation that is observed seems to correlate with the presence of serum during production is not surprising. Fetal bovine serum normally contains high concentrations of bovine plasminogen. The use of inhibitors to curtail this clipping has been described (Daiichi Seiyaku Co. Ltd, 1986), and the advantage afforded by their inclusion here is inferred from the high percentage of intact analogue.

These quantities of protein are more than adequate for all but the most consumptive analyses (e.g. kinetic studies, active site titrations, fibrin and other types of binding studies, development of purification strategies). By combining amplification with butyrate induction, sufficient quantities of material for more consumptive analyses, such as x-ray crystallographic studies (200–400 mg) can be produced simply and relatively inexpensively using 10 to 20 roller bottles in several months, depending on the expression level of the cells used (data not shown).

This work suggests obvious applications for butyrate in commercial scale processes, where extending the productive life of high density cultures would be of considerable value. Additionally, the expression-enhancing attribute of butyrate would directly reduce cost per gram of product.

Acknowledgements

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